



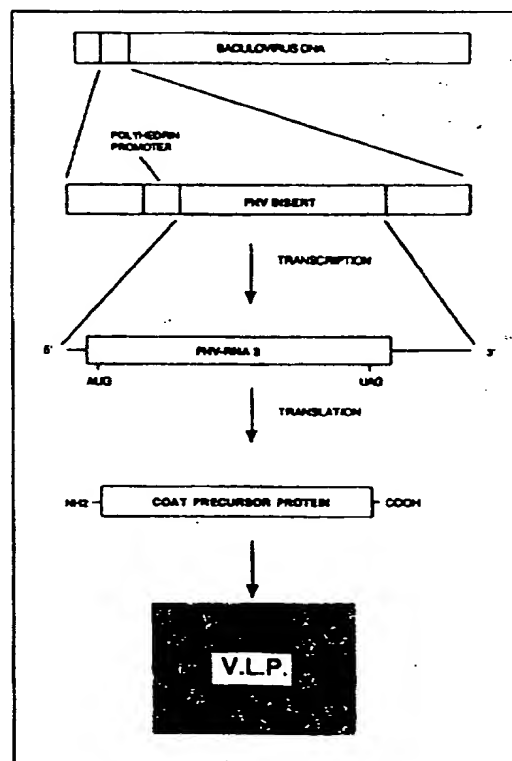
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(54) Title: **MOLECULAR PRESENTING SYSTEM**

(57) Abstract

The invention refers to a molecular presentation system in which viral proteins are foreseen as carriers for heterologous amino acid sequences. Hereby, the viral protein is derived from small insect viruses, primarily from Flock House Virus (FHV), with a known 3-dimensional structure and amino acid sequence, whereby heterologous amino acid sequences, for exemple epitopes, are inserted in the outwards directed loops of the viral capsid protein. Moreover, the expression of the FHV capsid protein in insect cells can produce mature virus like particles (VLP) through a recombinant baculovirus.

FHV Capsomer virus-like particles produced in Baculovirus.



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Molecular presenting system

The invention concerns a molecular presentation system in which viral proteins are being used as carriers for heterologous amino acid sequences.

The possibility to identify and synthesize amino acid sequences from viral proteins, which are able to generate a protective immune response in animals, has stimulated the development of synthetic vaccines. Although it has already been shown that synthetic peptides in some cases can induce a good immune response, it has turned out that in general they were weak immunogens unless coupled to strongly immunogenic carrier molecules. They were frequently unable to induce protective immunity in vaccinated animals. Attempts to increase the immunogenicity of these antigens for use as vaccine have lead to the development of a series of antigen presentation systems. Many of these are designed to present the antigen as a polyvalent, particulate structure. The development of particulate vector systems for immunogenic epitopes provides a powerful approach for the presentation of antigens. Various systems were used to present foreign epitopes: the core antigen of Hepatitis B virus (HBV) (HBcAg) [1] and the surface antigen of Hepatitis B virus (HBsAg) [2], the capsid protein from Polio virus [3], the yeast Ty protein [4], the particles obtained after insertion of HIV 1-gag in Baculovirus [5], rotavirus VP-6 protein [6], core particles of the Bluetongue virus (BTV) [7], and filamentous as well as icosahedral bacteriophages [8,9].

It has been demonstrated that the immunogenicity of a peptide depends on its sequence as well as on the way it is presented to the immune system. By using a human rhino virus capsid sequence as a heterologous peptide and the particles of HBcAg as a carrier, it was shown that the internal location of the foreign sequence increases the immunogenicity of the epitope by 10 to 50 fold when compared to the amino terminus location [10]. Also the antigenicity (measured as reactivity to a monoclonal antibody (mAb)) was greatly enhanced by placing the foreign peptide in that position in the carrier. Furthermore, both constructs presented the epitopes considerably more efficiently to the mAbs than the free peptides. This was also the case when specific HIV-1 epitopes (the V3 loop) were introduced into different domains of the HBcAg [11]. Since the properties

of a given epitope can be influenced by its conformation it was of great interest to have a carrier system with multiple entry sites conferring many possible conformations. This would increase the possibility of finding a conformation closer to the native one for a given sequence. In spite of the fact that, as mentioned above, various particulate systems have been developed for the presentation of epitopes, they were all based on the foreign epitope being inserted mainly in one position. This was partly due to lack of knowledge about the 3-D structure of the carrier particle.

Summary of the invention.

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With reference to the above, a new presentation system has been developed, characterized by the fact that the carrier protein is derived from small insect viruses, Flock House virus (FHV), with a known 3-D structure and amino acid sequence. Heterologous amino acid sequences, for example epitopes, are inserted into the outwards directed loops of the viral capsid protein. This carrier presents multiple possibilities for a conformationally suitable location of epitopes. Above all, the carrier system is characterized by the fact that the recombinant protein, or the virus like particles, are obtained from procaryotic or eucaryotic cells through the expression of the protein encoded by the appropriately modified RNA-2 gene of the FHV capsid protein.

20

Characteristics of the carrier particle.

Flock House Virus (FHV).

FHV is a non-enveloped icosahedral insect virus with a bipartite RNA genome and belonging to the Nodaviridae family. These viruses are among the smallest and simplest known. The FHV genome consists of two single stranded mRNA molecules (RNA-1 with 3.1 kb and RNA-2 with 1.4 kb), both encapsidated in the same particle. RNA-1 carries the information for the viral RNA-polymerase and RNA-2 codes for the coat precursor, alpha protein. Upon synthesis the coat precursor alpha is rapidly assembled with both RNAs, whereby immature, virion-like particles (provirions) are formed. These are slowly processed to mature particles by autocatalytic cleavages [12].

X-ray diffraction studies have shown the structure of the viral particles at 3.0 Angstrom resolution [13] (see fig 1). The virion has 60 icosahedral, asymmetric units each consisting of three quasiequivalent protomers forming a protein shell around the inner RNA genome [14]. The protomers consist of 1) a basic, crystallographically disordered aminotermius, 2) a Beta-barrel structure, 3) an outer protrusion composed predominantly of Beta sheets and formed by three large insertions between the strands of the Beta-barrel, and 4) a carboxyterminal domain composed of two distorted helices lying inside the shell. The external zone of the virion, which is the least conserved, has many sequence differences which essentially contain all the deletions and insertions of the 10 different strains [12]. The variations in the loops, directed outwards from the segments of the Beta-barrel structure, define serologically distinct viral strains.

These loops were selected as the regions to be manipulated for the insertion of the foreign epitopes. The positions for these insertions (L1, L2, L3, I1, I2, I3,) are given by the following amino acid regions of the RNA 2 gene:

| | | |
|----|---------|---------------------|
| 15 | Loop L1 | amino acids 195-219 |
| | Loop L2 | amino acids 263-277 |
| | Loop L3 | amino acids 129-138 |
| | Loop I1 | amino acids 107-110 |
| | Loop I2 | amino acids 152-165 |
| 20 | Loop I3 | amino acids 304-310 |

In fig. 2, showing the DNA sequence of FHV RNA-2 and the corresponding amino acid sequence, the individual loop regions are accentuated.

Fig. 3 shows the full restriction map of the DNA sequence of FHV RNA-2.

25 Fig. 4 represents the number of cutting sites of the endonucleases.

Fig. 5 shows all sites in which the endonucleases cut FHV RNA-2.

Fig. 6 is a graphic representation of the unique cutting sites of the endonucleases.

FHV grows vigorously in cultured cells and produces yields of 20% of the total cell protein [14]. In addition, FHV grows well in several Lepitopteran larvae. The viruses 30 of this family show a considerable resistance to inactivation by heat, detergents and other denaturants [14]. It was shown recently [15] that the expression of the capsid protein

FHV RNA-2 in insect cells via a recombinant baculovirus produces virion like particles (VLP) similar to authentic virions. It was shown by the present inventors that this can also be achieved by the expression of a modified gene carrying insertions for the expression of foreign amino acids within the capsomer structure. The VLPs generated by this procedure in insect cells are mature particles since the precursor protein, which is present in provirions, is cleaved. This system allows the production of 1 - 2 mg of purified synthetic virions (VLPs) in 50 ml of cultured cells [16].

Another method for the production of particles carrying foreign epitopes is by recovery of infectious virions after cotransfection of the genomic RNA-2 (obtained by in vitro transcription of modified cDNAs) with purified RNA-1 [17]. This is only valid for genomes which carry alterations that do not change the replicative cycle or the assembly of the virus. The RNA-1 can be purified by several cycles of autonomous replication in DM-1 cells (*Drosophila Melanogaster*) taking advantage of the fact that RNA-1 behaves as an autonomous replicon in transfected cells [18].

15

Detailed description of the construction of recombinant baculovirus carrying the wild type or modified capsomer gene.

FHV was grown in DM-1 cells and purified in sucrose and CsCl gradients as described in [19]. The genomic RNA was extracted from the purified virions by treatment with proteinase K and by phenol-chloroform extraction. A single stranded cDNA was made with reverse transcriptase using 20 bases long oligonucleotides complementary to the 3' end sequence [12] (see fig 2). A double stranded cDNA was made with standard PCR techniques [20] amplifying the single stranded cDNA using a 20 bases long oligonucleotide, complementary to the 5'-end of the RNA-2, together with the first primer. Both primers carried extra bases coding for selected restriction enzyme sites (Bam-HI site for the 5'-end and Xba-I site for the 3'-end). After the PCR amplification, the double stranded DNA was gel-purified and ligated to pUC18 (Sma I site). For the in vitro transcription of RNA-2 the corresponding cDNA was inserted into the plasmid pBluescript SKII (Stratagene) under the control of phage T7 polymerase. Examples of the above mentioned modifications of FHV RNA-2 are shown in table 1.

Table 1

| Position | Restriction Sites | Comments | | | | | | | | | | | | |
|------------------|------------------------------------|---|------|------|------|------|------------------|-----|------------------|-----|-----|-----|-----|-----|
| L 1 | Kpn I (after mutagenesis) | a) Aminoacids 205 to 209 (ATDPA) deleted from the original sequence. b) Val 204 mutagenized to Gly (GTT to GGT) to create Kpn I site: <table><tr><td>G204</td><td>T205</td></tr><tr><td>GGT</td><td>ACC</td></tr><tr><td>CCA</td><td>TGG</td></tr></table> c) After oligo insertion, GT duplicates | G204 | T205 | GGT | ACC | CCA | TGG | | | | | | |
| G204 | T205 | | | | | | | | | | | | | |
| GGT | ACC | | | | | | | | | | | | | |
| CCA | TGG | | | | | | | | | | | | | |
| L 2 | Pst I (after mutagenesis) | a) Aminoacids 270 to 273 (GSTG) deleted from the original sequence. b) Mutagenesis of the codon usage in L269 (CAG to CTG) and Q274 (CAA to CAG) to generate Pst I site: <table><tr><td>L269</td><td>Q274</td></tr><tr><td>CTG</td><td>CAG</td></tr><tr><td>GAC</td><td>GTC</td></tr></table> c) After oligo insertion, LQ duplicates. | L269 | Q274 | CTG | CAG | GAC | GTC | | | | | | |
| L269 | Q274 | | | | | | | | | | | | | |
| CTG | CAG | | | | | | | | | | | | | |
| GAC | GTC | | | | | | | | | | | | | |
| L 3 | Nhe I Spe I (original) | a) Aminoacids 128 to 134 (VPAGTFP) deleted after doble digestion with <u>Nhe I</u> - <u>Spe I</u> : <table><tr><td>A126</td><td>S127</td><td>T135</td><td>S136</td></tr><tr><td><u>Nhe I</u> GCT</td><td>AGC</td><td><u>Spe I</u> ACT</td><td>AGT</td></tr><tr><td>CGA</td><td>TCG</td><td>TGA</td><td>TCA</td></tr></table> b) S127 and T135 are regenerated after oligo insertion. There are no duplications of aa. | A126 | S127 | T135 | S136 | <u>Nhe I</u> GCT | AGC | <u>Spe I</u> ACT | AGT | CGA | TCG | TGA | TCA |
| A126 | S127 | T135 | S136 | | | | | | | | | | | |
| <u>Nhe I</u> GCT | AGC | <u>Spe I</u> ACT | AGT | | | | | | | | | | | |
| CGA | TCG | TGA | TCA | | | | | | | | | | | |
| I 3 | Bsu36 I (original) | a) There is no loss of aa in the original sequence. b) Oligo insertion duplicates aa P304 and E305 <table><tr><td>P304</td><td>E305</td><td>G</td></tr><tr><td>CCT</td><td>GAG</td><td>G</td></tr><tr><td>GGA</td><td>CTC</td><td>C</td></tr></table> | P304 | E305 | G | CCT | GAG | G | GGA | CTC | C | | | |
| P304 | E305 | G | | | | | | | | | | | | |
| CCT | GAG | G | | | | | | | | | | | | |
| GGA | CTC | C | | | | | | | | | | | | |
| I 2 | BamH I (after mutagenesis) | a) Aminoacids 154 and 155 (TT) are deleted after mutagenesis. b) Change of codon usage in S156 (TCA to TCC) to generate BamH I site: <table><tr><td>G153</td><td>S156</td></tr><tr><td>GGA</td><td>TCC</td></tr><tr><td>CCT</td><td>AGG</td></tr></table> c) GS duplicates after oligo insertion. | G153 | S156 | GGA | TCC | CCT | AGG | | | | | | |
| G153 | S156 | | | | | | | | | | | | | |
| GGA | TCC | | | | | | | | | | | | | |
| CCT | AGG | | | | | | | | | | | | | |
| I 1 | Bsu36 I (after mutagenesis) | a) Mutagenesis of G108 and Q109 to generate Bsu36 I site: <table><tr><td>G108</td><td>Q109</td></tr><tr><td>GGA</td><td>CAG</td></tr></table> to: P108 E109 CCT GAG G (cf D110) | G108 | Q109 | GGA | CAG | | | | | | | | |
| G108 | Q109 | | | | | | | | | | | | | |
| GGA | CAG | | | | | | | | | | | | | |

The cDNA of RNA-2 was also inserted into the vector pVL-1393 (Invitrogene) (Bam-HI/Xba-I sites). In this vector the gene is placed under the control of the polyhedrin promotor and flanked by sequences of the Autographa californica Nuclear Polyhedrosis Virus (AcNPV) which allow in vivo production of recombinant virus after cotransfection with AcNPV genomic DNA.

Introduction of foreign sequences in the cDNA of RNA-2

10 Small insertions/deletions in the sequences of RNA-2 were carried out by the PCR technique [20]. The epitope specific sequences were inserted into one or more of the selected sites either using restriction enzyme sites (when available) or by the PCR technique. The stereo diagram of the FHV capsid protein precursor in fig. 7 shows the sites where the specific HIV-1 sequences "IGPGRAF" were inserted. Those amino acids
15 were inserted into the positions L1, L2, L3, and I2, whereas the aminoacids "IGPGRAFE" were inserted into position I3. In all positions, except in position I3, certain amino acids were deleted: In position L1 amino acids 205 - 209 were deleted and aa 204 was mutated to create Kpn I site; in position L2 amino acids 270 - 273 were deleted and aa 269 and 274 were mutated to create Pst I site; in position L3 amino acids
20 128 - 134 were deleted after digestion with Nhe I-Spe I. In position I2 amino acids 154 - 155 were deleted. See table 1.

Examples of the insertion of foreign sequences into the recombinant FHV capsomer are listed in table 2.

25

30

TABLE 2

Examples of foreign sequences included in FHV recombinant capsomer

| Sequences | Sites | Amino acids sequence and their characteristics | Expressed in |
|-------------|-------------------|---|-----------------------|
| HBV-PreS1 | I3 | MGTNLSVPNPPAFGANST- -NPDWDFNPGGMQWNSTAL Tcell epitope. Receptor binding site. | E.coli |
| HBV-PreS2 | I3 | MQWNSTALDPRVRGL B cell epitope | E.coli |
| HBV-S | L1,L2,L3 I2,I3 | CTTPAQGNSMFPSCCCTKPTDGNC B cell epitope | E.coli Baculovirus |
| HCV-core | L1,L2,L3 I2,I3 | 1. TNPKPQRKTKRNTNRRPQD 2. VKFPGGGQIVGGVYLLPRR B cell epitopes. | E.coli Baculovirus |
| HIV-1 gp120 | L1,L2,L3 I2,I3 | IQRGPGRAF (IIIB) IGPGRAF (MN) FGPGQAL (Mal) IGPGRTL (NY5) KGPGRVI (RF) IGLGQAL (Z2) V3 loop.B cell epitope. Neutralizing epitope | E.coli Baculovirus |
| HIV-1 gp120 | L1,L2,L3 I2,I3 | 1.GKAMYAPPI 2. NMWQE(K)VGKA (C4).B cell epitope. Neutralizing epitope | E.coli Baculovirus |
| HIV-1 gp41 | L1,L2,L3 I2,I3 | ELDKWAS B cell epitope Neutralizing epitope | E.coli Baculovirus |
| HIV-1 gp41 | L1,L2,L3 I2,I3 | IEEEGGERDRDR B cell epitope Neutralizing epitope | E.coli Baculovirus |

Production of recombinant Baculovirus carrying the RNA-2 gene:

The cDNA of RNA-2 (wild-type or after genetic manipulation) was inserted into the transfer vector pVL-1393 under a polyhedrin promoter (sites Bam HI and XbaI of the polylinker). This pUC9 based vector carries a segment of AcNPV in the sequence flanking its polylinker and allows the transfer of the foreign gene to a baculovirus genome after in vivo recombination (see fig. 8). Insect cells (Spodoptera Frugiperda SF-21 cells) were co-transfected (LipoFectin) with linear genomic DNA (non-viable) of AcNPV (BaculoGold from PharMingen) and with the transfer vector carrying the FHV gene. After 4 days the virus progeny was harvested and titered. Thereafter, several recombinant viruses were plaque purified (3 to 4 times from well isolated plaques). These recombinants were denominated AcNPV-FHV. In some cases the VLPs can tolerate the insertion of up to 20 amino acids without alteration of the assembly process. In other cases, where the insertions prevented the formation of VLPs, this could be circumvented by coinfection with both the wild-type and the modified baculovirus. Thereby, mosaic VLPs were generated carrying both types of capsomer structures.

Production and purification of VLPs from insect cells infected with AcNPV-FHV.

20

In order to obtain purified antigens for immunological antigen studies, Sf-21 cells (in suspension or as monolayer) were infected with recombinant baculoviruses at a multiplicity of infection of 10. Two to three days after the infection 0.5% nonidet P-40 and 0.1% Beta Mercaptoethanol (2-ME) were added to the medium. After 15 minutes on ice, the cell debris were removed by centrifugation for 10 minutes at 12000 g. The VLPs in the supernatant were pelleted through a 30 wt/wt % sucrose cushion (50 mM HEPES, 0.1% 2-ME) at 40.000 rpm in an SW41 rotor for 3 hours at 4 C. The pellet was resuspended in 50 mM HEPES, 0.1% 2-ME and laid on a 10 ml 5-20 wt/wt % continuous sucrose gradient in the same buffer. The particles were sedimented in an SW41 rotor at 40.000 rpm for 1 hour at 11 C. The fractions of the gradient were collected from the bottom and aliquots of each fraction were run on a 10% SDS

poyacrylamid gel in order to localize the particle peak. The fractions containing the VLPs were pooled, pelleted by centrifugation and resuspended in the same buffer. The protein content of these preparations was determined by Micro BCA Protein Assay Reagent (Pierce).

5

Recovery of modified FHV containing exogenous sequences.

Live, recombinant FHV viruses can be recovered when the heterologous amino acid sequences which are inserted into the capsomer do not alter the virion assembly process.

10 The recovery was carried out by co-transfection of DM-1 cells with in vitro made transcripts of modified RNA-2 and authentic RNA-1 purified of RNA-2 by multiple transfection passages at the limiting dilution as described by Ball [18].

Example 1:

15 Production of VLPs carrying the HIV-1 (Human Immunodeficiency Virus, type 1) specific sequence IGPGRAF:

Several domains of the HIV-1 gp-120 can induce the production of neutralizing antibodies. One of them is the hypervariable region 3 (V-3 loop). This is a linear, 20 immunodominant epitope known as the "Principal Neutralizing Determinant" (PND) [21, 22]. Although the entire domain varies greatly among different isolates, it was recently found that, maybe due to conformation restraints, the amino acid sequence on the tip of the loop is well conserved. Sequence data from 245 isolates from the USA showed that the V3 loop sequence "GPGRAF" was present in more than 60% of the isolates [23]. In 25 addition, it was found that animals immunized with peptides containing this sequence produced sera which could neutralize several diverging isolates, although with a low titer [24]. This sequence was inserted into five different positions on the surface of the FHV structure and in some cases in two sites of the same molecule. The positions selected were the outwards directed loops mentioned above (see FHV structure fig.1). In one case 30 (position I3) the foreign sequence was introduced directly as an insert in the original sequence of RNA-2. In order to obtain this, the cDNA coding for the FHV capsidprotein

was digested with Bsu 36I (cuts the DNA at nucleotide 934), and a synthetic oligonucleotide was inserted coding for the HIV-1 specific sequence. As a consequence of this procedure an additional glutamic acid was inserted at the carboxyterminus of the HIV-1 sequence. The structures of all these recombinant proteins are shown in fig. 7.

5 Fig. 9 shows the proteins induced in Sf-21 cells after infection with the recombinant baculovirus carrying the HIV-1 epitopes in the positions shown in fig. 7. Cells infected with these recombinants, mock infected cells and cells infected with baculoviruses without inserts were lysed and analyzed in a 10% polyacrylamide gel. Coomassie staining of the gel showed, in the lysates from cells infected with recombinant
10 viruses, the presence of bands with a molecular weight similar to the expected molecular weight for FHV capsomer precursor protein (alpha protein) or its cleavage product. These bands were not present in the case of lysates from cells infected with baculovirus without the insert (AcNPV-RP6). Western blots from similar gels, analyzed with rabbit hyperimmune anti-FHV serum, confirmed the identity of the chimeric proteins. In
15 addition to the alpha precursor, its cleavage product (the mature beta protein) was seen in all cases. This probably indicates that the modified capsomers are still capable of assembling and autocleaving. However, in some cases the percentage of mature protein seemed to be low (e.g. L3; I3; I2), probably indicating that the presence of the insert affects the autocleavage process. When a similar blot was analyzed, either using sera
20 from HIV-1 positive patients or HIV-1 specific human monoclonal antibodies, a quite different pattern of recognition developed. The patients' sera mainly recognized the epitope in the L2 position or in those combinations where this position was used. On the contrary, the monoclonal antibodies strongly recognized the position L1 or combinations derived from that position. Position L3 was also extensively recognized by patients' sera
25 though consistently less than L2. The other positions were barely detectable by these sera. On the other hand, certain human sera detected preferentially proteins carrying the inserts in the positions L3 or I3. This suggests a difference in the specificity of the individual immune response to the same sequence. However, until now the strongest signals were always obtained when the proteins carried the inserts in the positions L1 or L2.
30 Coomassie staining of the gels showed that the differences do not depend upon the amount of induced protein in the insect cells. This confirmed the hypothesis that the

antigenicity of the epitope is influenced by its localization. Until now the reason for the differences in the patterns of recognition in different patients could not be explained. Further investigations are now to be carried out to explain these data. For example concerning the origin of the infecting strain, the neutralization titer of the sera, the differences in the idiotypic answers, the difference in the patients' prognosis, etc.

Purification of VLP-V3 from recombinant, baculovirus infected Sf-21 cells.

Fig.10 shows the sedimentation profile and the antigen composition of VLPs 10 produced by three different baculovirus: AcNPV-FHV which expresses the unmodified FHV capsid protein; AcNPV-FHV-V3/L1 expressing the same protein yet carrying the HIV-1 epitope in position L1; and AcNPV-FHV-V3/L2 carrying the insert in position L2. See fig. 7 for details on insert locations. In all cases it was found that the particulate material, obtained as described above, migrated to the same position in the gradient as the 15 FHV particles. The particulate nature of these components was further confirmed by electron microscopy. Aliquots from each peak were run on a polyacrylamide gel and probed with HIV-1 positive serum after transfer to nitrocellulose paper. In all cases the detected proteins migrated to the same position as did the FHV capsid protein or its precursor. In the case of wild-type or L1-derived particles the main band corresponded to 20 the mature protein, whereas in L2-derived particles a large quantity of immature protein (alpha protein) was present in addition. However, there seemed to be an increase of mature protein in VLPs when these were compared with the input material prior to the purification. Similar results were also obtained after analysis of the products of those recombinant baculoviruses expressing the FHV capsid proteins with the inserts in the 25 other positions described above. The only differences found were the yield of particulate material and the percentage of VLPs carrying immature protein in that material.

The experiments were carried out as follows. Four days after the infection the cells and the medium were processed as previously described and the presence of the particles was analyzed by sucrose density gradient sedimentation (SW50.1 rotor at 45000 30 rpm for 30 minutes at 20 degrees C). The fractions were collected from the bottom of the tubes. In order to detect the distribution of the FHV along the gradient, aliquots of each

fraction were tested for FHV reactivity by means of an ELISA assay. The distribution of HIV-1 specific reactivity along the gradient was measured through western blotting of samples from the peak fractions. The western blots were probed with HIV-1 positive sera and the resulting bands are shown at the bottom of each graph. In fig.10 graph a) shows the reactivity of AcNPV-FHV-derived particles, b) shows particles derived from AcNPV-FHV-V3-L1 and c) shows particles derived from AcNPV-FHV-V3-L2. The arrows indicate the migration of FHV run in a parallel gradient.

Immunogenicity of chimeric VLP-V3 particles

10

In order to determine whether the seven HIV-amino acids inserted into the VLP structure were capable of inducing an immune response, three groups of guinea pigs, each consisting of three animals, were immunized with purified VLPs carrying the HIV-1 insert as described in the following. The first group of animals was inoculated with the insert in position L3, the second group with the insert in position I3 and the third group was inoculated with the insert in both positions L3 and I3. All three groups were immunized subcutaneously with 500 microlitres PBS containing 50 microgrammes of the respective VLP preparations. For the first immunizations on day 0 the antigens were formulated in complete Freund's adjuvant (CFA). For the boosters on days 14 and 28 the same amount of antigen was formulated in Freund's incomplete adjuvant (IFA). Blood was taken from each animal 35 days after the first inoculation by cardiac puncture. Sera from the immunized animals were tested for specific anti-V3 and anti-FHV antibodies in an ELISA test. The data represent reciprocal dilutions at OD 492. For the anti-FHV titer the ELISA plates were coated with CsCl-purified viruses (200 nanogrammes per well). The titers against the HIV-1 inserts were analyzed on plates coated with recombinant gp120 (ABT-Baculovirus produced, 100 ng per well) as a capture antigen. The data in fig. 11 show that these preparations had elicited a good antibody response specific for the V3 sequence. As shown by this test, no major differences existed among the various constructs. However, the rest of the positions are yet to be analyzed and an evaluation is to be made of the differences in the affinity shown to the native gp-120 by the immune sera, a parameter known to be associated with their neutralizing capacity.

Example 2:

Hepatitis C Virus (HCV)

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The transfusion induced Hepatitis, which can neither be attributed to Hepatitis A virus nor to Hepatitis B virus (NANBH), belongs to the main group of transfusion transmitted diseases [25]. The cloning and expression of HCV has allowed the development of antibody screening immunoassays for the detection of HCV infections, 10 using as solid phase antigen a fusion polypeptide expressed through recombinant yeast. Initial studies using this protein confirmed that HCV was the predominant agent of NANBH. However, these and subsequent studies demonstrated a series of shortcomings with this serological test due to low sensitivity and specificity. The tests currently used are mostly based on the detection of antibodies against the non-structural proteins 15 NS3-NS4 which, however, do not appear in infected patients until the disease is in an advanced state (4-6 months after the onset of the Hepatitis). Later, it was demonstrated that most immunodominant epitopes are located within the aminoterminal parts of the core protein [25, 26, 27] and that antibodies against these epitopes appear early after the infection. This was shown either by using recombinant HCV-core protein produced in 20 bacteria or baculovirus, or by using synthetic peptides corresponding to these sequences. Moreover, the HCV-core protein is considered as the most significant single antigen for the detection of antibodies in infected patients. Among all positive samples 80-85 % are found to be core positive and in most of them this was the only antigen recognized.

25

Production of FHV-VLPs carrying an HCV-core epitope.

With reference to these considerations, the epitopes of the HCV core protein were tested in the molecular presentation system of the invention here reported. A 20 amino acids long sequence was selected corresponding to the amino acids 20-40 in the original 30 sequence about which it had already been shown that they were very effective for diagnostic purposes [26]. The 20 amino acids long epitope was inserted in the I3 position

on the Bsu-36I site of the RNA-2 gene contained in pVL-1393. The recombinant Baculovirus was produced and purified as described. The resulting recombinant was denominated AcNPV-HCVc. Fig. 12 shows the expression of hybrid FHV-HCV proteins through the recombinant baculovirus AcNPV-HCVc. The chimeric capsomer was produced through the recombinant baculovirus as follows. Sf-21 cells were infected with recombinant baculovirus AcNPC-HCVc (lane 3, 6, and 7), with a baculovirus carrying an unmodified FHV capsid protein AcNPV-FHV (lane 2 and 4) and with a polyhedrin-minus baculovirus carrying no insert AcNPV-RP6 (lane 1), respectively. The whole cell extracts were run in a 10% SDS-Page gel. Purified FHV was included as a marker (lane 5). Lanes 1-3 were stained with Coomassie blue. After the running, the proteins in lanes 4-7 were blotted on Nitrocellulose paper. After staining with Poinceau red, paper strips corresponding to each well were cut out and probed with a specific serum. Lanes 4 and 6 were probed with serum from a patient who was core positive in a RIBA-II test. Lanes 5 and 7 were probed with rabbit-anti-FHV serum.

15 The insect cells were infected with the recombinant virus and four days after the infection the cells were lysed and analyzed on a 10% SDS-PAGE gel. After the running, the gel was stained with Coomassie brilliant blue. The introduction of HCV-sequences apparently had no influence on the protein production. However, all the detected protein migrated with the molecular weight of the precursor (alpha protein). This indicates that the maturation process is somewhat impaired by the sequence alteration. In order to confirm the identity of this protein, the lysates of the infected cells were run on a similar gel, transferred to Nitrocellulose paper and probed with specific antisera. As expected, the protein reacted strongly with the specific rabbit-anti-FHV antiserum (dilution 1:2000) as well as with HCV positive human serum (dilution 1:200).

25 Fig.13 shows the FHV reactivity (measured in an ELISA test) after sucrose sedimentation of VLPs produced by infection of Sf-21 cells with the recombinant baculovirus AcNPV-HCVc. The running conditions were identical to those described in connection with fig.10. Aliquots from each peak fraction were western blotted and probed with HCV positive human sera. A photo of the developed Western bands is inserted at the bottom of the graph. The arrow indicates the position of FHV run in a parallel gradient.

As in the case of the particles carrying HIV-1 specific sequences, the particles migrated somewhat slower than the wild-type FHV particles. Western blots of aliquots from the peak reacted with HCV-positive sera. This indicates that the unprocessed protein is not impaired in its ability to autoassemble into a particulate structure.

5 To assess the capability of the antigen to detect specific antibodies, purified VLPs were used for the ELISA test. Wells of ELISA plates (Nunc) were coated with 100 microlitres of purified VLPs diluted in PBS buffer (100 nanogrammes per well). After blocking with PBS containing 5% BSA, 100 microlitres of serum dilution were added to each well and the plate was incubated for two hours at room temperature. The bound
10 antibodies were detected by a second incubation with a horse radish peroxidase conjugate of the IgG fraction of goat anti-human immunoglobulin for one hour at room temperature. The enzyme activity was measured using o-phenyldiamine as a substrate. The absorbance of each well was measured at $A = 490$ nanometer. To test the sensitivity of this antigen, 100 sera, known to be core positive in a commercial test (RIBA II-Chiron
15 Corp.), were analyzed. Almost 85% of the samples gave titres higher as 1:1000 which indicates a very good sensitivity when detecting anti-core antibodies. These results demonstrated that these 20 amino acids from the HCV core sequence represent a very reliable antigen for the detection of HCV infections, when introduced in the carrier system of the present invention.

20 The results are represented in the block diagram in fig. 14.

Comparison of a VLP-based ELISA test with current, commercially available tests.

In order to test the sensitivity of this ELISA assay, a collection of serially drawn
25 blood samples from infected patients, encompassing the period of seroconversion, were analyzed for specific HCV core antibodies. At that stage, all patients already have a high level of the specific liver enzyme Alanine aminotransferase (ALT). In four out of 50 patients which were analyzed (see fig. 15) the test subject of this invention showed seroconversion earlier than in the currently available testkits (RIBA II). These results
30 show that the antigen is extremely suitable for detection of contaminated samples in blood banks. The serially drawn blood samples from selected patients, taken for the RIBA-II

test before the seroconversion, were analyzed by using plates coated with VLPs carrying an HCV core-specific epitope. The serum dilution was 1:100 for RIBA-II as well as for the ELISA test of the invention here reported. The RIBA-II values are shown in the upper panel. The VLP-based ELISA test values are represented in the diagram as circles, the 5 ALT values as a vertical line.

A comparison between antibody detection by VLPs, carrying HCV core sequences, and antibody detection by the free HCV peptide.

10 It has been shown that short peptides are very efficient when used as capture antigens for detection of specific antibodies in human as well as animal sera especially in the form of branched peptides [29]. It has also been reported that they react better than the corresponding recombinant antigens [30]. In transfusion induced Hepatitis-C cases it was established that by using peptides as capture antigens, positive sera could be detected
15 as early as one month after the first transfusion. This coincides with the first increase in the specific liver enzymes and would make short amino acid sequences a useful marker for detecting acute specific HCV infections [27]. For this reason it was decided to compare in a dot-blot assay the HCV specific antigens described in the invention at issue with the corresponding free peptides (HCc-2p), with a peptide encompassing the first 20
20 amino acids of the core (HCc-1p), and with peptides corresponding to other HCV proteins (NS peptides), respectively.

Fig. 16 shows a photograph of these dot-blots which were carried out as follows. Aliquots of 10 microlitres of purified VLPs (5 microgrammes per ml), carrying an insert of 20 amino acids from the core of HCV, and solutions containing peptides representing
25 different areas of the HCV genome (100 microgrammes per ml) were blotted on nitrocellulose paper. After blocking with 5% fat free and dry milk, each strip was incubated with a 1:100 dilution of human sera. After washing, each filter was incubated with anti-human antibodies conjugated to horse radish peroxidase (Dako, dilution 1:5000), and finally incubated with diaminobenzidine (DAB). Lanes 1 - 6 show patients'
30 sera. Lane NC shows the negative control serum.

As can be seen, already very low levels of antigen (50 nanogrammes corresponding to 2.5 nanogrammes of the specific HCV peptide) are strong enough, in the form of VLPs, to elicit a good signal with a positive sample. The corresponding free peptide (HCc-2p) gave only a very weak signal although it recognized the same number 5 of positive samples. In this case, the amount of antigen loaded onto the nitrocellulose paper was 400 times higher as in the case of the VLPs, based on a molar ratio. The peptide corresponding to the first 20 amino acids (HCc-1p) gave stronger signals, but failed to detect one positive sample and gave an indeterminate result with another positive sample. Peptides which corresponded to other HCV proteins and which were designed on 10 the basis of published results [31] are far less efficient for detection of HCV positive sera.

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CLAIMS:

1. A molecular presentation system in which viral proteins are used as carriers for 5 specific amino acid sequences and which is characterized by the fact that the viral protein is derived from small insect viruses of known 3-dimensional structure and amino acid sequence - preferentially from FHV - in which heterologous amino acid sequences, such as epitopes, are inserted into the outwards directed loops of the capsid protein.
2. A system according to claim 1, characterized by the fact that a recombinant 10 protein, or virus like particle, is used as viral protein whereby this recombinant protein, or virus like particle, is obtained from procaryotic or eucaryotic cells through the expression of the protein encoded by the appropriately modified RNA-2 gene of the FHV capsid protein, as shown in figure 2.
3. A system according to claim 2, characterized by the fact that the heterologous 15 amino acid sequences are inserted into one or more regions of the outwards directed loops of the FHV capsid protein, encoded by the RNA-2 gene, in which the loops denoted L1,L2,L3,I1,I2,I3 are chosen.
4. A system according to claim 3, characterized by the fact that the regions of the loops selected for the insertion of the heterologous amino acid sequences are defined by 20 the following amino acid sequence regions of the FHV Capsid protein encoded by the RNA-2 gen:

Loop L1 amino acid sequence region 195 - 219
Loop L2 amino acid sequence region 263 - 277
25 Loop L3 amino acid sequence region 129 - 138
Loop I1 amino acid sequence region 107 - 110
Loop I2 amino acid sequence region 152 - 165
Loop I3 amino acid sequence region 304 - 310
- 30 5. A process for the production of a molecular presentation system according to claims 3 or 4 characterized by the fact that the RNA-2 gene of the FHV capsid protein, in

the regions corresponding to loops L1, L2, L3, I1, I2, I3, is modified by deletions and/or mutagenesis and/or insertions in order to create cut sites for restriction enzymes in the said loop regions that can be used for the inserts which encode the heterologous amino acid sequences.

- 5 6. Application of a molecular presentation system according to one of the claims 1 to 4 for therapeutic, diagnostic and immunization purposes.

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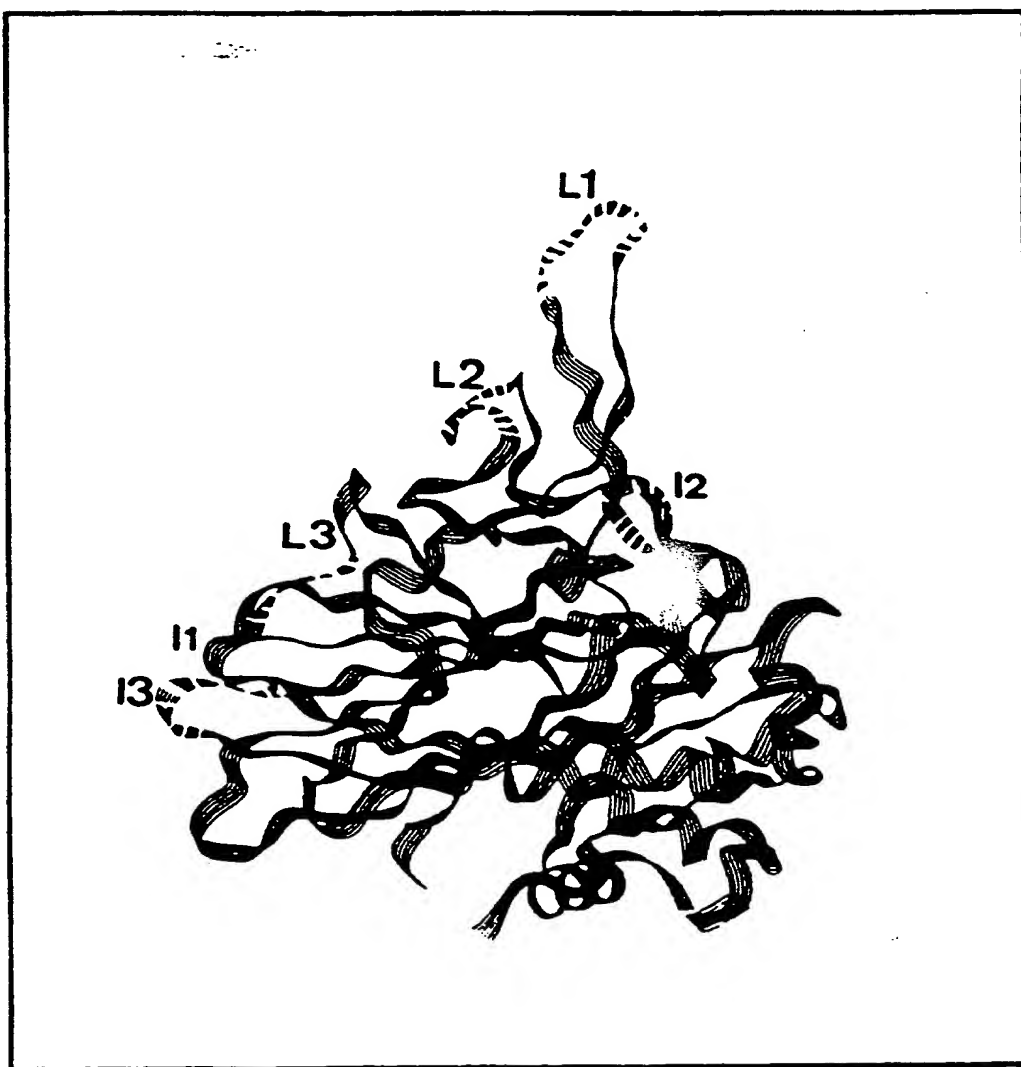
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FIG.1 Crystallographic representation of the outward directed loops of the FHV Capsid protein precursor with 3 Å resolution, showing the positions of insertions of the foreign genes (L1, L2, L3, I1, I2, I3).



23

53

atggttaataacaacagaccaagacgtcaacgagctcaacgcgttgctcgtcacaacaacc
M V N N N R P R R Q R A Q R V V V T T T

83

113

caaacagcgcctgttccacagcaaaacgtgccacgtaatggtagacgccgacgtaatcgc
Q T A P V P Q Q N V P R N G R R R R N R

143

173

acgaggcgtaatcgccgacgtgtgcgcggaatgaacatggcggcgctaaccagattaagt
T R R N R R R V R G M N M A A L T R L S

203

233

caacctgggttggcggttctcaaagtgtgcatttgcaccacctgacttcaacaccgacccc
Q P G L A F L K C A F A P P D F N T D P

263

293

ggtaaggggaataacctgatagatttgaaggcaaagtggtcagccgaaaggatgtcctcaat
G K G I P D R F E G K V V S R K D V L N

323

353

caatctatcagctttactgccggacaggacacttttatactcatcgcacctacccccgga
Q S I S F T A G Q D T F I L I A P T P G

383

413

gtcgctactggagtgctagcgttcctgctgggtacttttctactagtgcgactacgttt
V A Y W S A S V P A G T F P T S A T T F

443

473

aaccccggttaattatccgggttttacatcgatgttcggaacaacttcaacatctagggtcc
N P V N Y P G F T S M F G T T S T S R S

503

533

gatcaggtgtcctcattcaggtacgttccatgaacgtgggtatttaccacaacgtcgaac
D Q V S S F R Y A S M N V G I Y P T S N

563

593

ttgatgcagtttgccggaagcataactgtttggaaatgccctgtaaagctgagtactgtg
L M Q F A G S I T V W K C P V K L S T V

623

653

caattcccgggttgcaacagatccagccaccagttcgctagttcataactcttgttggttta
Q F P V A T D P A T S S L V H T L V G L

683

713

gatgggtgttctagcgggtggggcctgacaacttctctgagtcattcatcaaaggagtgttt
D G V L A V G P D N F S E S F I K G V F

743

773

tcacagtcggcttgtaacgagcctgactttgaattcaatgacatattggaggggtatccag
S Q S A C N E P D F E F N D I L E G I Q

803

833

acattgccacctgctaattgtgtcccttggttctacgggtcaaccttttaccatggactca
T L P P A N V S L G S T G Q P F T M D S

863

893

ggagcagaagccaccagtggagtagtcggatggggcaatatggacacgattgtcatccgt
G A E A T S G V V G W G N M D T I V I R

923

953

gtctcggcccctgagggcgcagtttaactctgccataactcaaggcatggtcctgcattgag
V S A P E G A V N S A I L K A W S C I E

983

1013

tatcgaccaaattccaaacgccatgttataccaattcggccatgattcgcctcctctcgat
Y R P N P N A M L Y Q F G H D S P P L D

1043

1073

gaggtcgcgcttcaggaataccgtacgggttgccagatctttgccgggttgcaagtgatagcg
E V A L Q E Y R T V A R S L P V A V I A

1103 1133
gccccaaatgcatcaatgtgggagagagtgaaatccatcattaaatcctccctggctgct
A Q N A S M W E R V K S I I K S S L A A

1163 1193
gcaagcaacattcccggcccgatcggtgtcgccgcaagtggattagtggaactgtcagcc
A S N I P G P I G V A A S G I S G L S A

1223 1253
ctttttgaaggatttggcttttagaagcatccggacgccaacctaaccgggcaagtatcc
L F E G F G F STOP

1283 1313
gaacaatcggacatttggccacaataagcccaatttggttgaagattaaagtagtgagcc

1343 1373
cccttagcgcgaaaccggaatttatattccaaaccagtttaagtcaacagactaagg

Restriction Endonucleases site usage

| | | | | | | | |
|----------|---|----------|---|----------|----|----------|---|
| Aat II | 1 | Bsp1286I | 2 | Fse I | - | Pac I | - |
| Acc I | 1 | Bsp1407I | - | Fsp I | - | Paer7 I | - |
| Acc65 I | - | BspD I | 1 | Gdi II | 1 | PflM I | 1 |
| Aci I | 5 | BspE I | 1 | Hae I | 1 | Ple I | 3 |
| Afl II | - | BspH I | - | Hae II | 2 | Pme I | - |
| Afl III | 2 | BspM I | 1 | Hae III | 6 | Pml I | - |
| Age I | - | BspW I | 4 | Hga I | 2 | Ppu10 I | 1 |
| Alu I | 3 | Bsr I | 4 | HgiA I | 1 | PpuM I | - |
| Alw I | 1 | BsrF I | 1 | Hha I | 6 | PshA I | - |
| AlwN I | 1 | BssH II | - | Hinc II | 5 | Psp1406I | - |
| Apa I | - | Bst1107I | - | Hind III | - | Pst I | - |
| ApaL I | - | BstB I | - | Hinf I | 4 | Pvu I | 1 |
| Apo I | 2 | BstE II | - | HinP I | 6 | Pvu II | - |
| Asc I | - | BstK I | 8 | Hpa I | 1 | Rsa I | 4 |
| Ase I | - | BstN I | 2 | Hpa II | 11 | Rsr II | - |
| Ava I | - | BstU I | 4 | Hph I | - | Sac I | 1 |
| Ava II | 2 | BstX I | 1 | Kas I | - | Sac II | - |
| Avr II | - | BstY I | 2 | Kpn I | - | Sal I | - |
| BamH I | - | Bsu36 I | 1 | Mae II | 8 | Sap I | - |
| Ban I | - | Cla I | 1 | Mae III | 2 | Sau3A I | 4 |
| Ban II | 2 | Csp6 I | 4 | Mbo I | 4 | Sau96 I | 6 |
| Bbe I | - | Dde I | 6 | Mbo II | 1 | Sca I | 1 |
| Bbs I | - | Dpn I | 4 | Mcr I | 1 | ScrF I | 8 |
| Bbv I | 2 | Dpn II | 4 | Mlu I | 1 | SfaN I | 3 |
| BceF I | - | Dra I | - | Mme I | 1 | Sfc I | - |
| Bcg I | 1 | Dra III | - | Mnl I | 9 | Sfi I | - |
| Bcl I | - | Drd I | 2 | Msc I | 1 | SgrA I | - |
| Bcn I | 6 | Dsa I | 1 | Mse I | 8 | Sma I | - |
| Bfa I | 5 | Dsa V | 8 | Msp I | 11 | SnaB I | - |
| Bgl I | - | Eae I | 2 | Mun I | - | Spe I | 1 |
| Bgl II | 1 | Eag I | - | Nae I | - | Sph I | - |
| Bpm I | 1 | Eam1105I | - | Nar I | - | Srf I | - |
| Bpu1102I | - | Ear I | - | Nci I | 6 | Sse8337I | - |

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| | | | | | | | |
|---------|---|-----------|---|----------|---|-----------|---|
| Bsa I | - | Ecl136 I | 1 | Nco I | 1 | Ssp I | - |
| BsaA I | 1 | Eco47 III | - | Nde I | - | Stu I | - |
| BsaB I | 1 | Eco57 I | 1 | NgoM I | - | Sty I | 2 |
| BsaH I | 3 | EcoN I | 1 | Nhe I | 1 | Swa I | - |
| BsaJ I | 5 | EcoO109 I | 1 | Nla III | 6 | Taq I | 4 |
| BsaW I | 2 | EcoR I | 1 | Nla IV | 2 | Tfi I | 1 |
| Bsg I | - | EcoR II | 2 | Not I | - | Tth111 I | - |
| BsiE I | 1 | EcoR V | - | Nru I | - | Tth111III | 3 |
| BsiW I | 1 | Ehe I | - | Nsi I | 1 | Xba I | - |
| Bsl I | 6 | Esp3 I | - | Nsp I | - | Xcm I | - |
| Bsm I | - | Fau I | - | Nsp7524I | - | Xho I | - |
| BsmA I | 1 | Fnu4H I | 5 | NspB II | - | Xma I | - |
| Bspl20I | - | Fok I | 4 | NspC I | - | Xmn I | 1 |

Enzyme/Recognition sequence/n.sites/positions:

| | | | |
|---------|--------------|---|------|
| Aat II | gacgt/c | 1 | 45 |
| Acc I | gt/mkac | 1 | 123 |
| Alw I | ggatc 4/5 | 1 | 641 |
| AlwN I | cagn3/ctg | 1 | 87 |
| Bcg I | cgan6tgc | 1 | 558 |
| Bgl II | a/gatct | 1 | 1076 |
| Bpm I | ctggag 16/14 | 1 | 391 |
| BsaA I | yac/gtr | 1 | 114 |
| BsaB I | gatnn/nnatc | 1 | 910 |
| BsiE I | cgry/cg | 1 | 1182 |
| BsiW I | c/gtacg | 1 | 1064 |
| BsmA I | gtctc 1/5 | 1 | 923 |
| BspD I | at/cgat | 1 | 469 |
| BspE I | t/ccgga | 1 | 1252 |
| BspM I | acctgc 4/8 | 1 | 811 |
| BsrF I | r/ccggy | 1 | 1084 |
| BstX I | ccan5/ntgg | 1 | 531 |
| Bsu36 I | cc/tnagg | 1 | 932 |
| Cla I | at/cgat | 1 | 469 |

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| | | | |
|-----------|--------------|---|------|
| Dsa I | c/crygg | 1 | 852 |
| Ecl136 I | gag/ctc | 1 | 54 |
| Eco57 I | ctgaag 16/14 | 1 | 1052 |
| EcoN I | cctnn/n3agg | 1 | 513 |
| EcoO109 I | rg/gnccy | 1 | 700 |
| EcoR I | g/aattc | 1 | 773 |
| Gdi II | yggccg -5/-1 | 1 | 1018 |
| Hae I | wgg/ccw | 1 | 1298 |
| HgiA I | gwgwc/c | 1 | 54 |
| Hpa I | gtt/aac | 1 | 944 |
| Mbo II | gaaga 8/7 | 1 | 1323 |
| Mcr I | c/grycg | 1 | 1182 |
| Mlu I | a/cgcgt | 1 | 61 |
| Mme I | tccrac 20/18 | 1 | 887 |
| Msc I | tgg/cca | 1 | 1298 |
| Nco I | c/catgg | 1 | 852 |
| Nhe I | g/ctagc | 1 | 398 |
| Nsi I | atgca/t | 1 | 1110 |
| PflM I | ccan4/ntgg | 1 | 113 |
| Ppu10 I | a/tgcat | 1 | 1110 |
| Pvu I | cgat/cg | 1 | 1182 |
| Sac I | gagct/c | 1 | 54 |
| Sca I | agt/act | 1 | 614 |
| Spe I | a/ctagt | 1 | 425 |
| Tfi I | g/awtc | 1 | 1025 |
| Xmn I | gaann/nnttc | 1 | 480 |

| | | | |
|----------|------------|---|-----------|
| Afl III | a/crygt | 2 | 61 160 |
| Apo I | r/aatty | 2 | 773 1360 |
| Ava II | g/gwcc | 2 | 498 969 |
| Ban II | grgcy/c | 2 | 54 1338 |
| Bbv I | gcagc 8/12 | 2 | 1157 1160 |
| BsaW I | w/ccggw | 2 | 1252 1356 |
| Bsp1286I | gdgch/c | 2 | 54 1338 |
| BstN I | cc/wgg | 2 | 206 1153 |
| BstY I | r/gatcy | 2 | 640 1076 |

| | | | |
|-----------|-------------|---|---------------------------|
| DrdI | gacn4/nngtc | 2 | 39 905 |
| Eae I | y/ggccr | 2 | 1018 1298 |
| EcoR II | /ccwgg | 2 | 206 1153 |
| Hae II | rgcgc/y | 2 | 88 184 |
| Hga I | gacgc 5/10 | 2 | 126 1256 |
| Mae III | /gtnac | 2 | 71 756 |
| Nla IV | ggn/ncc | 2 | 700 928 |
| Sty I | c/cwgg | 2 | 826 852 |
| | | | |
| Alu I | ag/ct | 3 | 55 332 609 |
| BsaH I | gr/cgyc | 3 | 45 126 1256 |
| Ple I | gagtc 4/5 | 3 | 381 719 857 |
| SfaNI | gcatc 5/9 | 3 | 565 1112 1249 |
| Tth111III | caarca 11/9 | 3 | 83 589 1164 |
| | | | |
| BspWI | gcn5/nngc | 4 | 103 1091 1103 1340 |
| BsrI | actgg 1/-1 | 4 | 390 651 876 1376 |
| BstU I | cg/cg | 4 | 62 167 1048 1350 |
| Csp6 I | g/tac | 4 | 414 523 615 1065 |
| Dpn I | ga/tc | 4 | 503 641 1077 1183 |
| Dpn II | /gatc | 4 | 503 641 1077 1183 |
| FokI | gcatg 9/13 | 4 | 310 890 916 1250 |
| Hinf I | g/antc | 4 | 381 719 857 1025 |
| Mbo I | /gatc | 4 | 503 641 1077 1183 |
| Rsa I | gt/ac | 4 | 414 523 615 1065 |
| Sau3AI | /gatc | 4 | 503 641 1077 1183 |
| Taq I | t/cga | 4 | 470 557 985 1038 |
| | | | |
| Aci I | ccgc -3/-1 | 5 | 168 182 695 1100 1194 |
| Bfa I | c/tag | 5 | 399 426 495 659 692 |
| BsaJ I | c/cnngg | 5 | 259 376 826 852 1152 |
| Fnu4H I | gc/ngc | 5 | 182 1100 1157 1160 1193 |
| Hinc II | gty/rac | 5 | 48 201 840 944 1385 |
| | | | |
| Bcn I | ccs/gg | 6 | 260 377 458 628 1175 1269 |
| Bsl I | ccn5/nngg | 6 | 113 259 260 371 513 919 |

| | | | |
|---------|--------|---|-----------------------------|
| Dde I | c/tnag | 6 | 611 717 859 933 1345 1394 |
| Hae III | gg/cc | 6 | 702 928 1019 1102 1178 1299 |
| Hha I | gcg/c | 6 | 89 166 185 939 1049 1349 |
| HinP I | g/cgc | 6 | 89 166 185 939 1049 1349 |
| Nci I | cc/sgg | 6 | 260 377 458 628 1175 1269 |
| Nla III | catg/ | 6 | 178 532 853 966 1003 1022 |
| Sau96 I | g/gncc | 6 | 498 701 928 969 1102 1178 |

| | | | |
|--------|--------|---|------------------------------------|
| BstK I | c/cngg | 8 | 206 260 377 458 628 1153 1175 1269 |
| Dsa V | /ccngg | 8 | 206 260 377 458 628 1153 1175 1269 |
| Mae II | a/cgt | 8 | 46 108 115 133 160 437 537 554 |
| Mse I | t/taa | 8 | 27 197 441 450 945 1143 1328 1381 |
| ScrF I | cc/ngg | 8 | 206 260 377 458 628 1153 1175 1269 |

| | | | |
|------|----------|---|---|
| MnlI | cctc 7/7 | 9 | 145 316 513 791 935 1031 1034 1043 1149 |
|------|----------|---|---|

| | | | |
|--------|-------|----|--|
| Hpa II | c/cgg | 11 | 261 342 378 458 576 629 1085 1176 1253 1269 1357 |
| Msp I | c/cgg | 11 | 261 342 378 458 576 629 1085 1176 1253 1269 1357 |

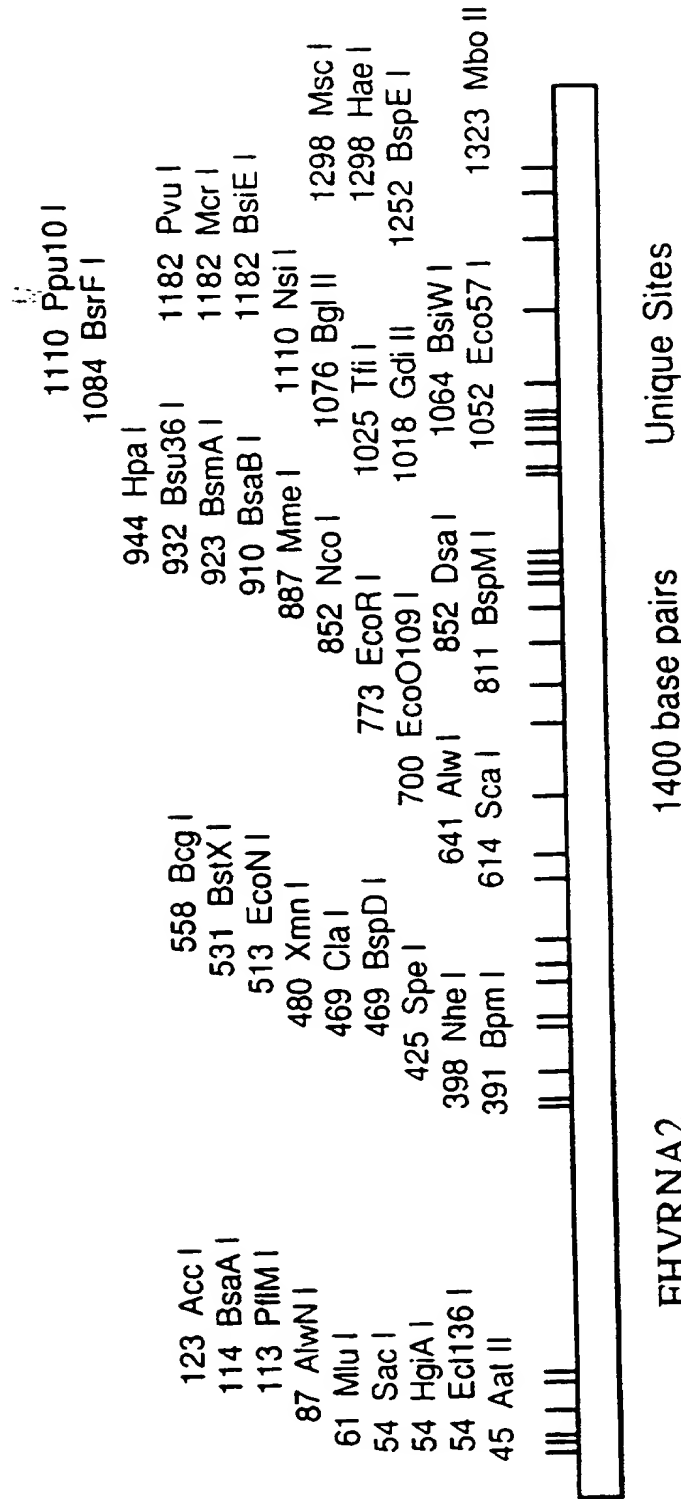
292 sites found

No Sites found for the following Restriction Endonucleases

| | | | | | |
|--------|-----------|----------|-------------|----------|-------------|
| Acc65I | g/gtacc | DraIII | cacn3/gtg | PmeI | gttt/aaac |
| AflIII | c/ttaag | EagI | c/ggccg | PmlI | cac/gtg |
| AgeI | a/ccggt | Eam1105I | gacn3/nngtc | PpuMI | rg/gwccy |
| ApaI | gggcc/c | EarI | ctcttc 1/4 | PshAI | gacnn/nngtc |
| ApaLI | g/tgcac | Eco47III | agc/gct | Psp1406I | aa/cggt |
| AscI | gg/cgcgcc | EcoRV | gat/atc | PstI | ctgca/g |
| AseI | at/taat | EheI | ggc/gcc | PvuII | cag/ctg |
| AvaI | c/ycgrg | Esp3I | cgtctc 1/5 | RsrII | cg/gwccg |
| AvrII | c/ctagg | FauI | cccgcc 4/6 | SacII | ccgc/gg |

| | | | | | |
|----------|-------------|----------|-----------|----------|--------------|
| BamHI | g/gatcc | FseI | ggccgg/cc | SalI | g/tcgac |
| BanI | g/gyrcc | FspI | tgc/gca | SapI | gctcttc 1/4 |
| BbeI | ggcgc/c | HindIII | a/agctt | SfcI | c/tryag |
| BbsI | gaagac 2/6 | HphI | ggtga 8/7 | SfiI | ggccn4/nggcc |
| BceFI | acggc12/13 | KasI | g/gcgcc | SgrAI | cr/ccggyg |
| BclI | t/gatca | KpnI | ggtac/c | SmaI | ccc/ggg |
| BglI | gccn4/nggc | MunI | c/aattg | SnaBI | tac/gta |
| Bpu1102I | gc/tnagc | NaeI | gcc/ggc | SphI | gcatg/c |
| BsaI | ggtctcl/5 | NarI | gg/cgcc | SrfI | gccc/gggc |
| BsgI | gtgcagl6/14 | NdeI | ca/tatg | Sse8337I | cctgca/gg |
| BsmI | gaatgc 1/-1 | NgoMI | g/ccggc | SspI | aat/att |
| Bsp120I | g/ggccc | NotI | gc/ggccgc | StuI | agg/cct |
| Bsp1407I | t/gtaca | NruI | tcg/cga | SwaI | atatt/aaat |
| BspHI | t/catga | NspI | rcatg/y | Tth111I | gacn/nngtc |
| BssHII | g/cgcgc | Nsp7524I | r/catgy | XbaI | t/ctaga |
| Bst1107I | gta/tac | NspBII | cmg/ckg | XcmI | ccan5/n4tgg |
| BstBI | tt/cgaa | NspCI | rcatg/y | XhoI | c/tcgag |
| BstEII | g/gtnacc | PacI | ttaat/taa | XmaI | c/ccggg |
| DraI | ttt/aaa | Paer7I | c/tcgag | | |

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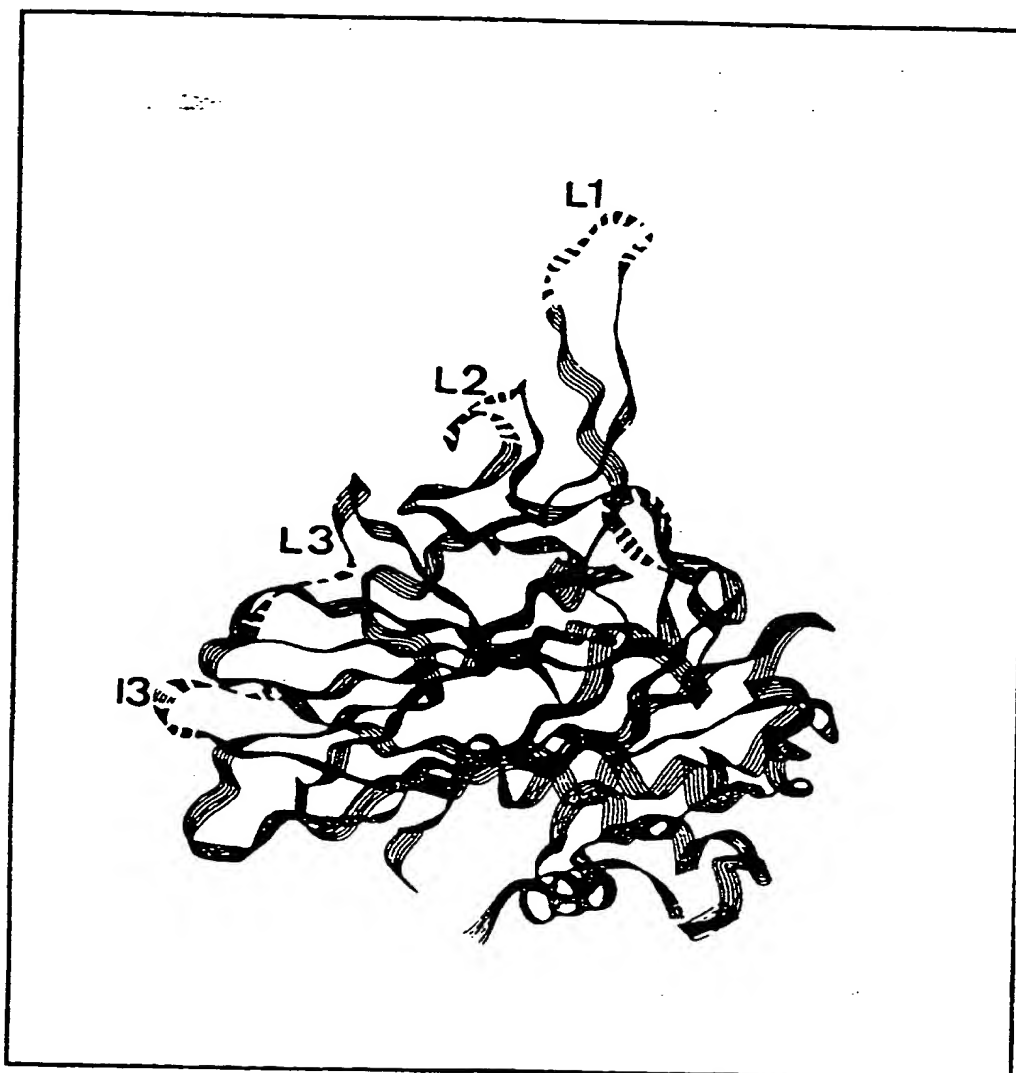
Unique Sites

1400 base pairs

FHVRNA2

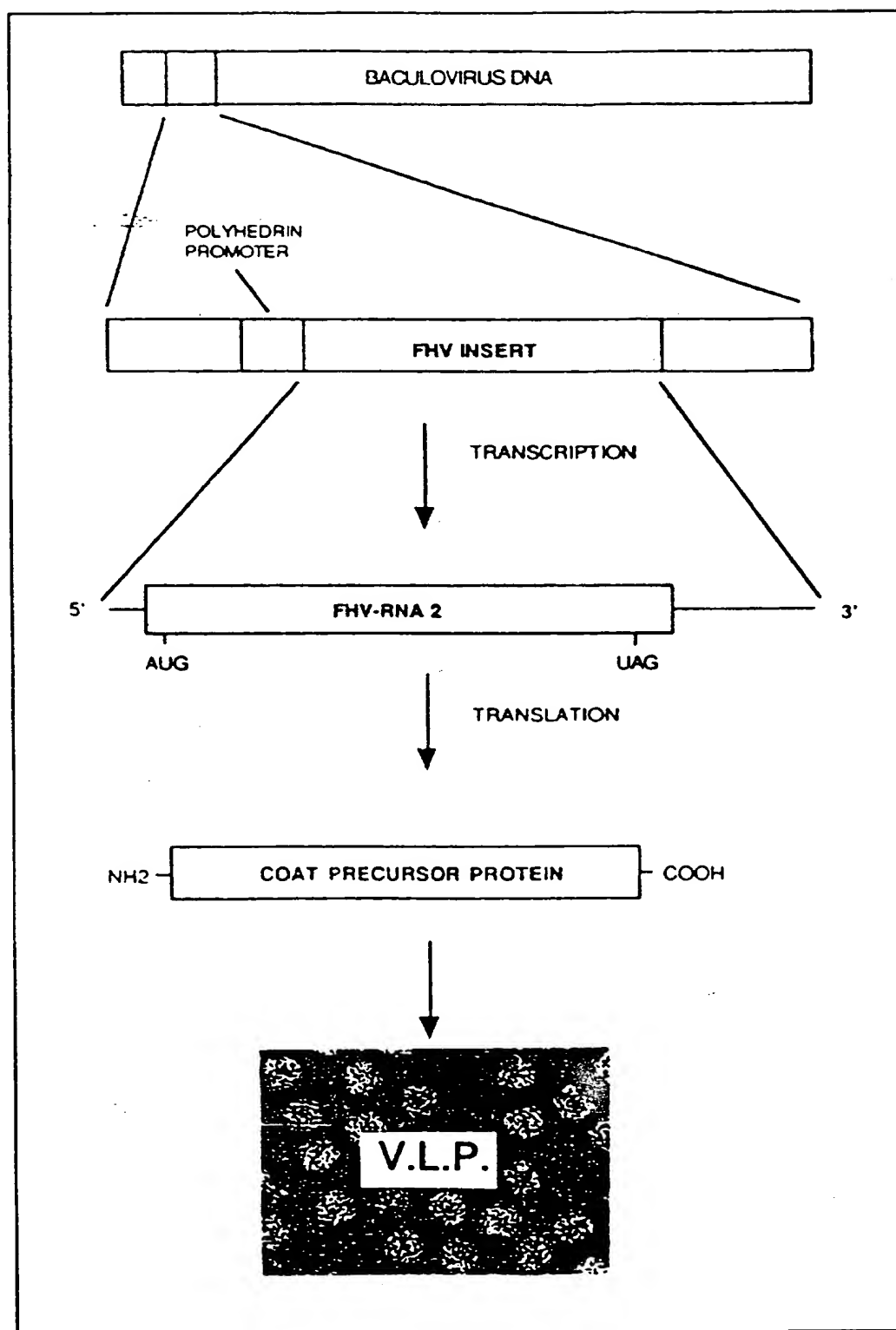
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FIG. 7 Stereo diagram of the FHV protein showing the positions in which the HIV-1 specific sequence "IGPGRAF" is inserted.



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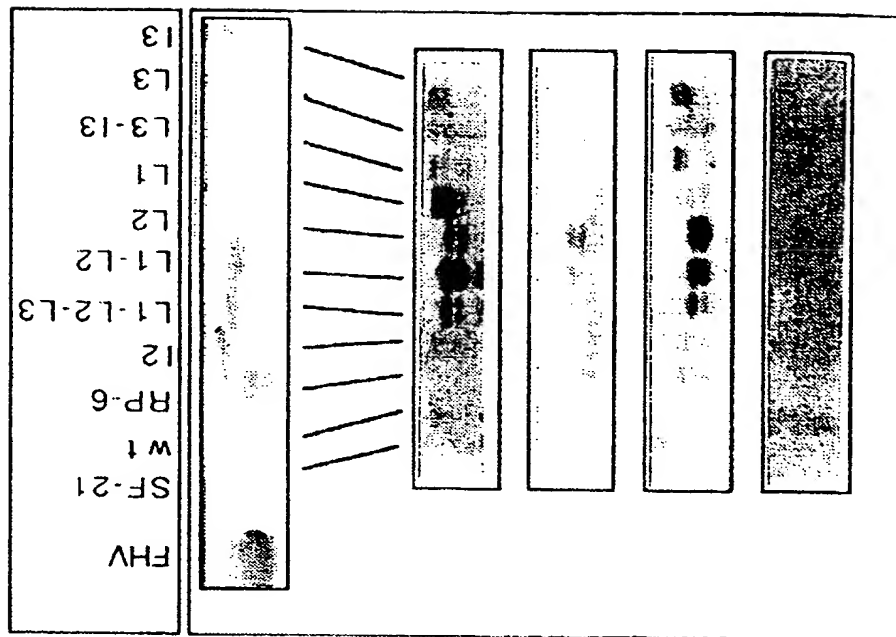
FIG. 8 FHV Capsomer virus-like particles produced in Baculovirus.



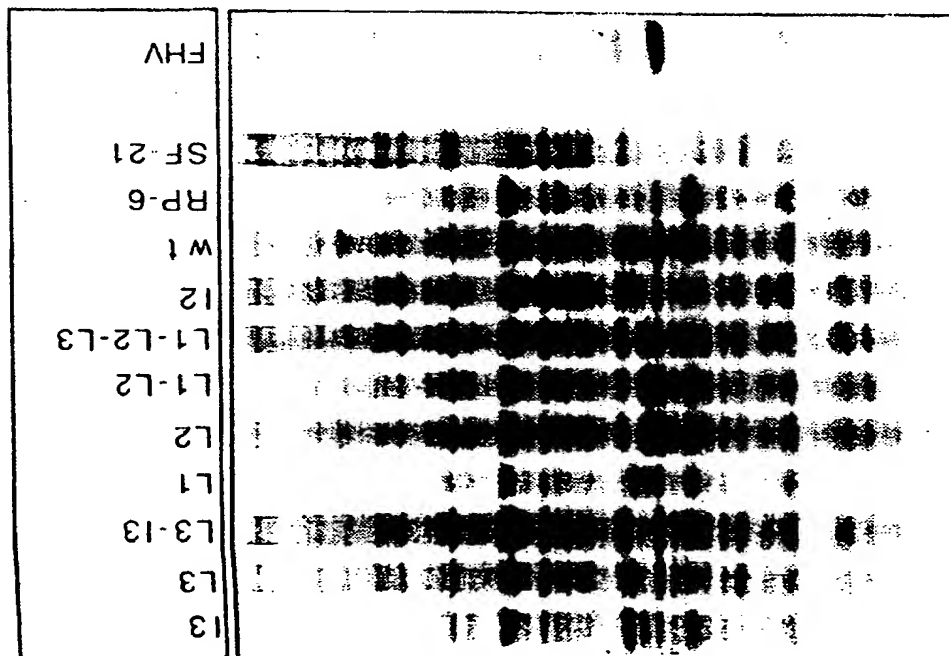
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FIG. 9 Expression of the wild-type and the hybrid FHV capsomer. SF-21 infected cell lysates were analyzed in 10% SDS-PAGE. **A)** Coomassie stained. **B)** Western blot analyzed with: (1) antiserum against FHV. (2) human HIV-1 mAb. (3 and 4) HIV-1 positive sera and (5) HIV-1 negative serum.

B)

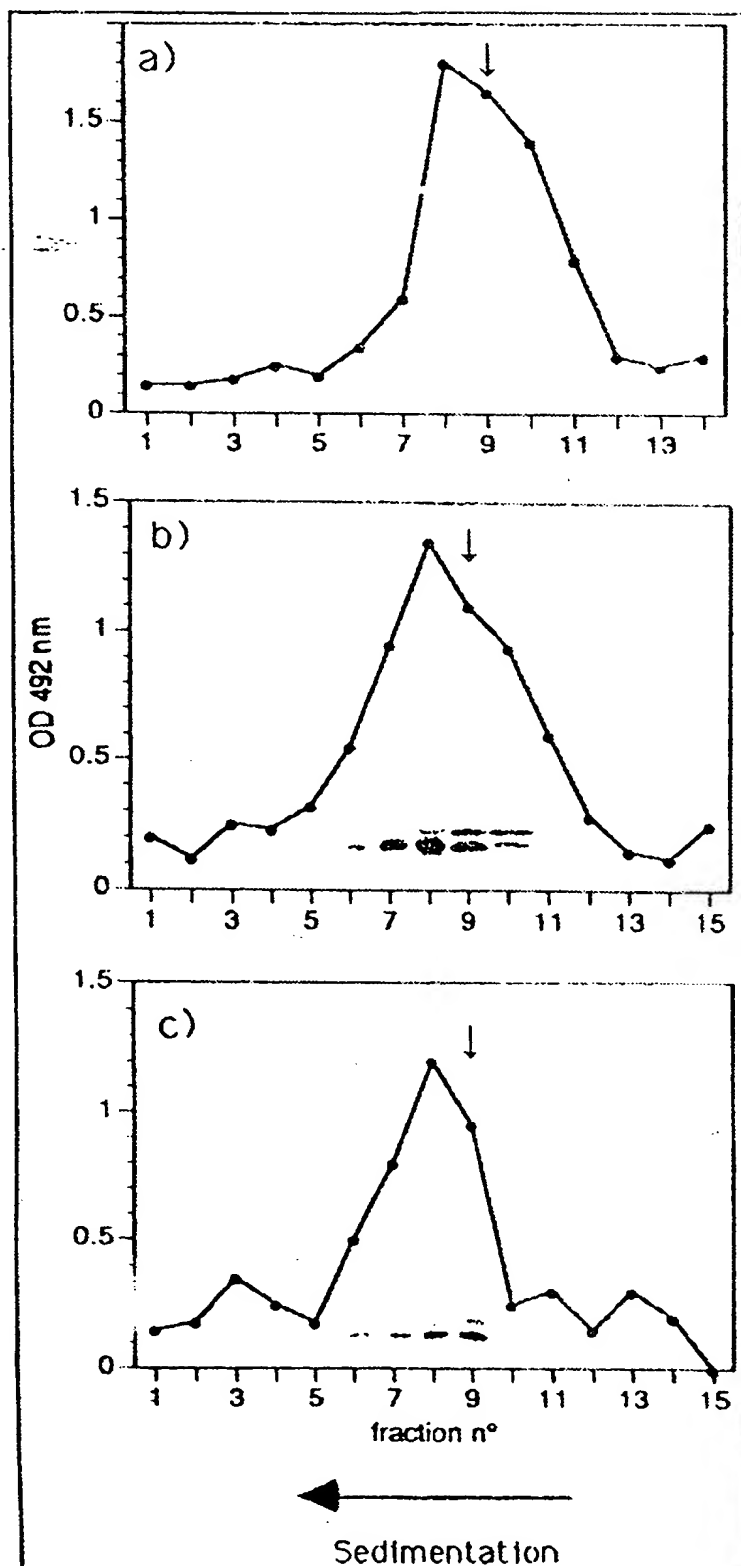


A)



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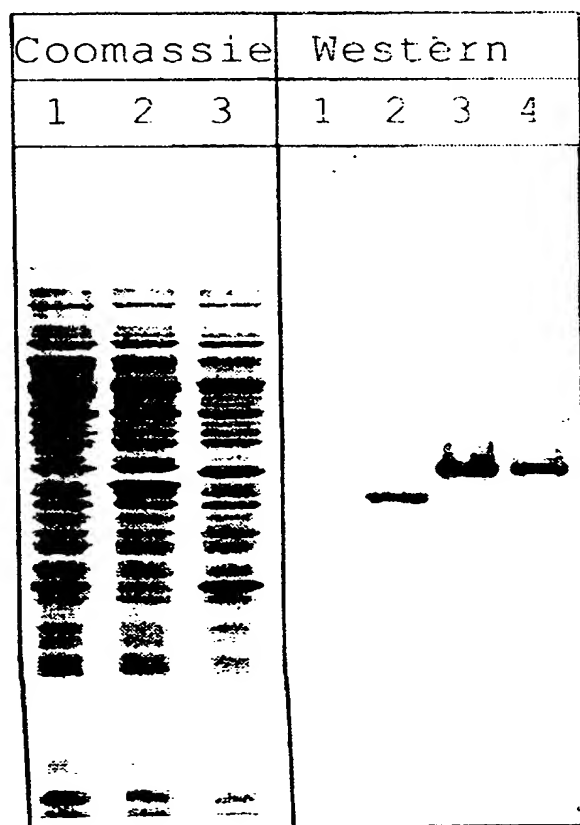
FIG.10 Sedimentation profile and antigen composition of VLPs produced by three different recombinant Baculovirus infected SF-21 cells.



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Fig.11 Serum anti-V3 antibody titres measured against recombinant gp120

| Ag. for Ab titre | Ac NPV-V3 L3 | Ac NPV-V3 I3 | Ac NPV-V3 I3-L3 |
|------------------------|-----------------|-----------------|--------------------|
| FHV | 1:40000 | 1:20000 | 1:5000 |
| | 1:20000 | 1:60000 | 1:15000 |
| | 1:30000 | 1:10000 | 1:20000 |
| gp 120 | 1:500 | 1:2000 | 1:250 |
| | 1:1500 | 1:5000 | 1:2000 |
| | 1:2000 | 1:1000 | 1:500 |

FIG.12 Expression of hybrid FHV-HCVc protein through recombinant Baculovirus AcNPV-HCHc.

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FIG.13 Profile and FHV reactivity (ELISA test) after a sucrose gradient of VLPs formed by SF-21 infected cells with the recombinant Baculovirus AcNPV-HCV.

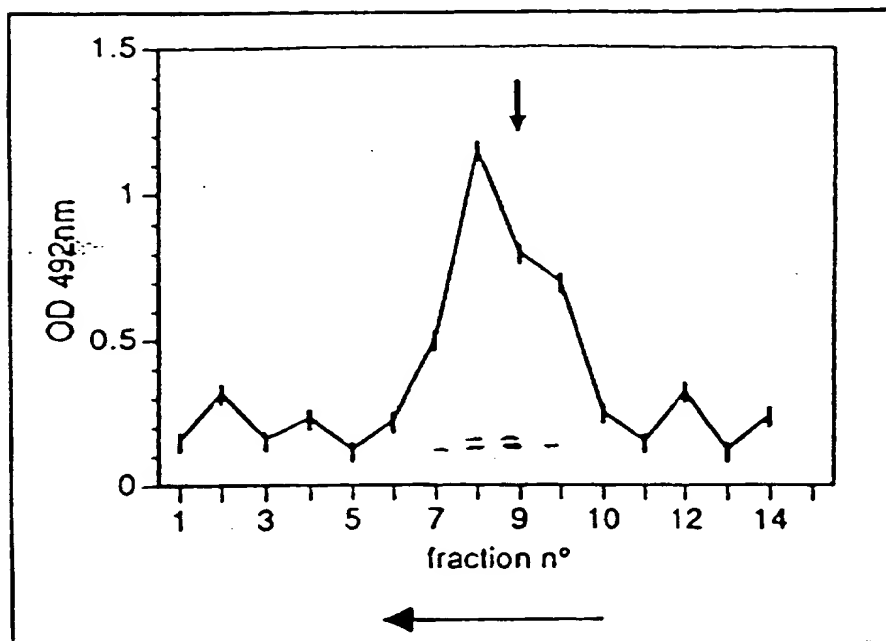
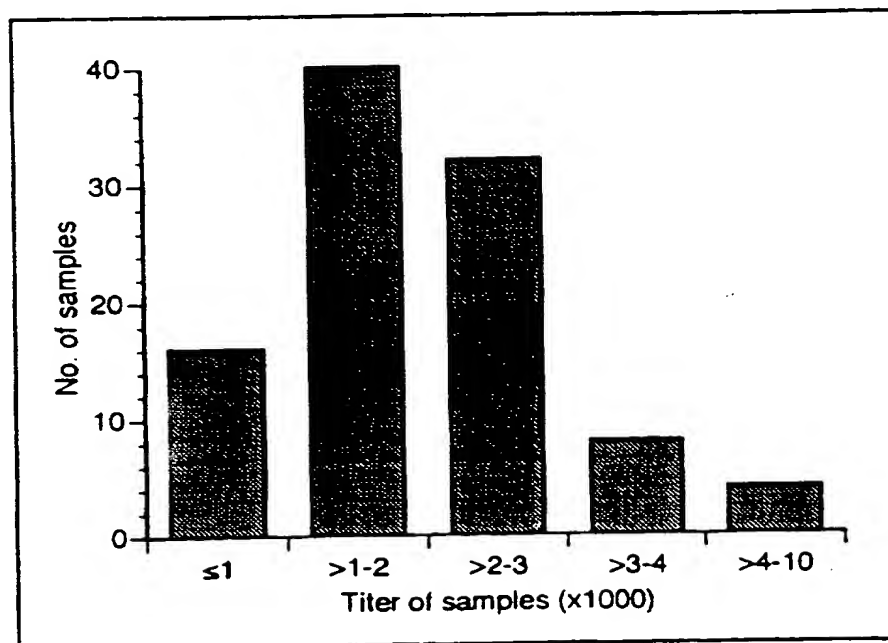
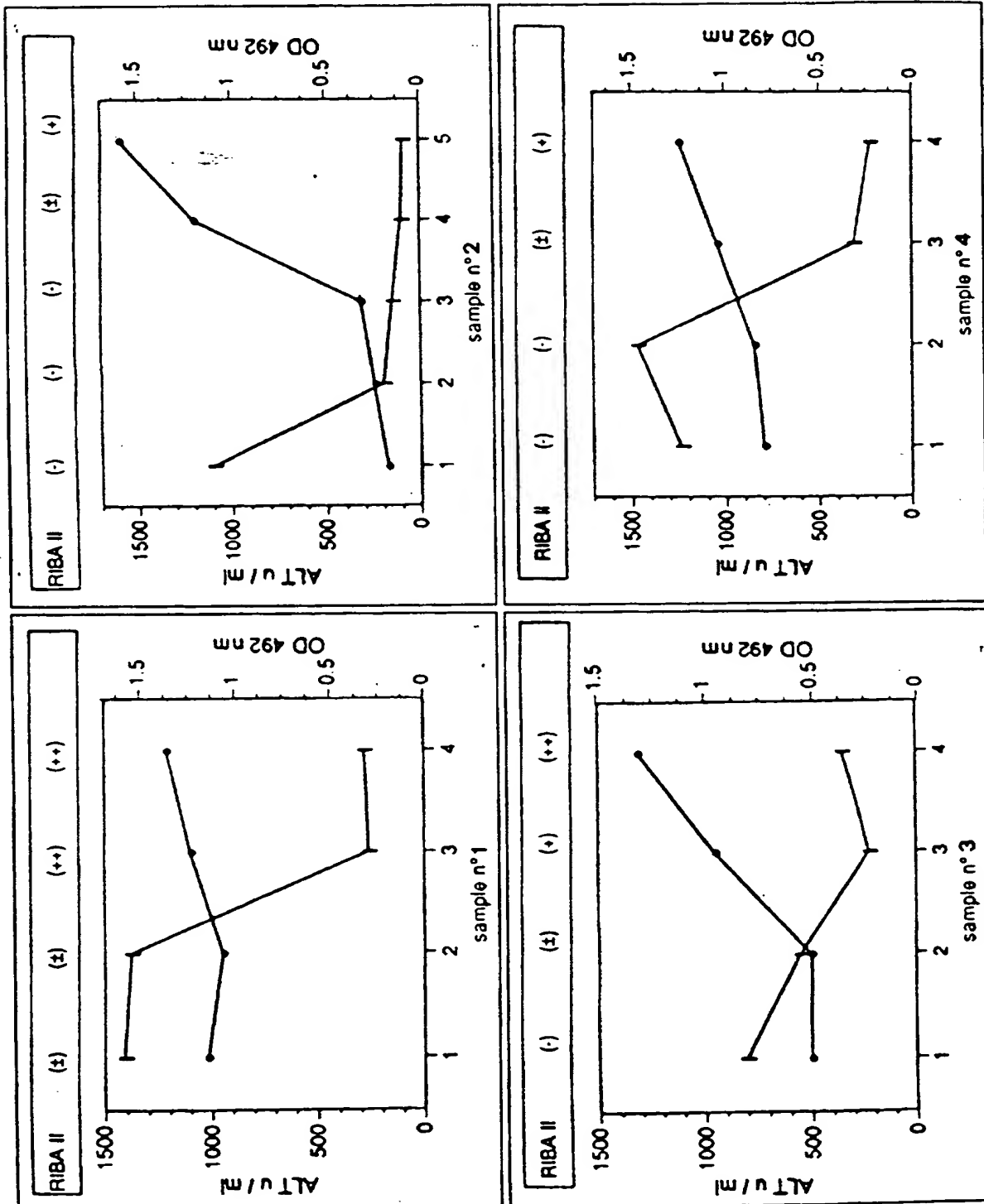


FIG.14 Distribution of the ELISA titre obtained by a VLP based assay among 100 selected HCV core positive sera.










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FIG.15 Detection of HCV specific antibodies in a VLP based ELISA test and its correlation with ALT levels and previous RIBA II values.



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FIG. 16 Detection of HCV core-antibodies in human sera by dot-blots using recombinant (VLP-HCV) antigen or free peptides.

| Patients | | | | | | | Antigen |
|--|--|--|--|--|--|---|---------|
| 1 | 2 | 3 | 4 | 5 | 6 | NC | |
|  |  |  |  |  |  |  | NS5-p |
| | | | | | | | NS4-p |
| | | | | | | | NS3-1-p |
| | | | | | | | NS3-2-p |
| | | | | | | | NS3-3-p |
| | | | | | | | HCc-1-p |
| | | | | | | | HCc-2-p |
| | | | | | | | pHCc-2 |

| | | |
|---|--|---|
| A. CLASSIFICATION OF SUBJECT MATTER | | |
| IPC 6 | C12N7/00 C07K14/18 | C12N15/86 A01N39/12 C12N15/62 G01N33/53 C07K14/06 07K14/16 |
| According to International Patent Classification (IPC) or to both national classification and IPC | | |
| B. FIELDS SEARCHED | | |
| Minimum documentation searched (classification system followed by classification symbols) IPC 6 C12N C07K A61K G01N | | |
| Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched | | |
| Electronic data base consulted during the international search (name of data base and, where practical, search terms used) | | |
| C. DOCUMENTS CONSIDERED TO BE RELEVANT | | |
| Category * | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
| Y | J. VIROL. (1993), 67(5), 2950-3 CODEN: JOVIAM; ISSN: 0022-538X, May 1993 FISHER, ANDREW J. ET AL 'Crystallization of viruslike particles assembled from flock house virus coat protein expressed in a baculovirus system' see the whole document --- | 1,2 |
| Y | J. VIROL. (1993), 67(5), 2756-63 CODEN: JOVIAM; ISSN: 0022-538X, May 1993 SCHNEEMANN, ANETTE ET AL 'Use of recombinant baculoviruses in synthesis of morphologically distinct viruslike particles of flock house virus, a nodavirus' see the whole document --- | 1,2 |
| | --- -/- | |
| <input checked="" type="checkbox"/> Further documents are listed in the continuation of box C. <input type="checkbox"/> Patent family members are listed in annex. | | |
| * Special categories of cited documents: "A" document defining the general state of the art which is not considered to be of particular relevance "E" earlier document but published on or after the international filing date "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) "O" document referring to an oral disclosure, use, exhibition or other means "P" document published prior to the international filing date but later than the priority date claimed "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art "&" document member of the same patent family | | |
| Date of the actual completion of the international search 4 January 1996 | | Date of mailing of the international search report 26. 01. 96 |
| Name and mailing address of the ISA European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HY Rijswijk Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl. Fax (+ 31-70) 340-3016 | | Authorized officer Hornig, H |

| C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT | | |
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| Category | Citation of documents with indication, where appropriate, of the relevant passages | Relevant to claim No. |
| Y | J. VIROLOGY, vol. 66, no. 7, July 1992 AM.SOC.MICROBIOL., WASHINGTON, US, pages 4003-4012, B. ROVINSKI ET AL. 'Expression and characterization of genetically engineered human immunodeficiency virus-like particles containing modified envelope glycoproteins: Implications for development of a cross-protective AIDS vaccine' see the whole document --- | 1,2 |
| Y | J. GENERAL VIROLOGY, vol. 74, no. 7, July 1993 READING, BERKS, GB, pages 1261-1269, R. WAGNER ET AL. 'Induction of cytolytic T lymphocytes directed towards the V3 loop of the human immunodeficiency virus type 1 external glycoprotein gp120 by p55gag/V3 chimeric vaccinia viruses' see the whole document --- | 1,2 |
| A | NATURE, vol. 361, 14 January 1993 MACMILLAN JOURNALS LTD., LONDON, UK, pages 176-179, A.J. FISHER AND J.E. JOHNSON 'Ordered duplex RNA controls capsid architecture in an icosahedral animal virus' see the whole document --- | 1-6 |
| A | NUCLEIC ACIDS RES. (1989), 17(18), 7525-6 CODEN: NARHAD; ISSN: 0305-1048, 25 September 1989 DASGUPTA, RANJIT ET AL 'Nucleotide sequences of three Nodavirus RNA2's: the messengers for their coat protein precursors' see the whole document --- | 1-6 |
| P,X | FEBS LETT. (1994), 353(1), 1-4 CODEN: FEBLAL; ISSN: 0014-5793, October 1994 TISMINETZKY, SERGIO G. ET AL 'Immunoreactivity of chimeric proteins carrying the HIV-1 epitope IGPGRF. Correlation between predicted conformation and antigenicity' see the whole document --- | 1-6 |
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INTERNATIONAL SEARCH REPORT

International Application No

PCT/EP 95/03114

C(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

| Category | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|----------|--|-----------------------|
| T | <p>VACCINE 13 (13). 1995. 1233-1239. ISSN: 0264-410X, September 1995 SCODELLER E A ET AL 'A new epitope presenting system displays a HIV-1 V3 loop sequence and induces neutralizing antibodies.' see the whole document -----</p> | 1-6 |

